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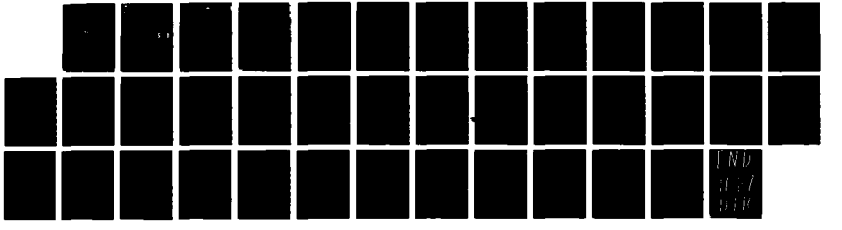

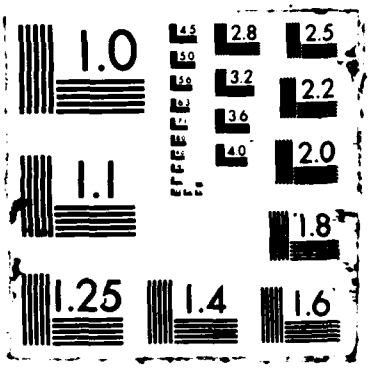
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PATHOGENESIS OF DENGUE VACCINE VIRUSES IN MOSQUITOES

Annual Report

Barry J. Beaty, Ph.D.

January 1, 1984

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The dengue-1 candidate vaccine (TP-56, nonmutagenized) and its parent virus were compared for their ability to infect orally Aedes aegypti and Aedes albopictus mosquitoes. The vaccine virus was as infective orally as the parent virus for both mosquito species (First Annual Report). Of the A. albopictus mosquitoes orally infected with parent and vaccine virus, 13% (2/16) and 5% (1/19) respectively, transmitted virus.

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Increasing extrinsic incubation temperature from 27° to 33°C generally increased infection and transmission rates of both parent and vaccine temperature treatment groups. Fifty percent (3/6) of the mosquitoes infected with parent virus and incubated at 27°C transmitted, while 67% (4/6) infected with vaccine virus transmitted. Parent and vaccine virus infected mosquitoes incubated at 33°C transmitted virus 50% and 38% of the time respectively (Table 2).

Dissection and examination by immunofluorescence of major organ systems of mosquitoes orally infected with parent and vaccine virus indicated that early in the infections (7 days), antigen was limited to the midgut tissues in both 27°C and 33°C treatment groups. At 14 days post-feeding, virus had disseminated from the midgut to secondary target organs. In addition, mosquitoes incubated at 33°C seemed to contain more detectable antigen in a wider variety of tissues than vaccine or parent virus infected mosquitoes incubated at 27°C.

The dengue-1 vaccine virus candidate, 45AZ5, was phenotypically stable after up to 3 passages in A. albopictus and A. aegypti mosquitoes. Studies are continuing on its ability to infect, replicate in, and be transmitted by mosquitoes.

The dengue-4 candidate vaccine virus H-241, Lot1, was less efficient than its parent virus in infection and replication in both A. aegypti and A. albopictus mosquitoes. A. albopictus mosquitoes seemed to be a more competent vector than A. aegypti. The median oral infectious dose (OID_{50}) for A. albopictus mosquitoes orally ingesting parent virus was 4.6 $\log_{10}TCID_{50}$ per ml and >7.0 logs for vaccine virus. For parent and vaccine virus in A. aegypti the OID_{50}'s were 5.0 and >8.0 logs respectively. After oral infection, the vaccine virus replicated at a slower rate and to a lower titer than the parent virus in both mosquito species. Again, incubation of mosquitoes at 33°C tended to result in faster replication of parent virus. In addition, mosquitoes incubated at the higher temperature contained greater amounts of virus and viral antigen early in the infection. Limited transmission data indicates that vaccine virus is transmitted less efficiently than parent in both mosquito species.

The dengue-4 candidate vaccine virus, AGd6, after multiple passages in C6/36 cell culture and adult mosquitoes, has proven to be unable to grow in C6/36 tissue culture, the basis of our assay system. Another candidate will be substituted.

Initial studies indicate that a primary infection with dengue-1 virus may interfere with other strains of dengue virus. However, this primary infection does not affect replication of a related flavivirus, West Nile, or unrelated bunyavirus, La Crosse.

SUMMARY

Studies were conducted to compare the efficiency of oral infection, replication and oral transmission of dengue-1 and dengue-4 candidate vaccine viruses and their respective parent viruses in vector mosquitoes.

The dengue-1 candidate vaccine virus, TP-56 was not altered in its ability to infect and to replicate in Aedes aegypti or Aedes albopictus mosquitoes. Oral infection and dissemination rates with the vaccine virus were as high or higher than for the parent virus. Transmission rates of vaccine infected mosquitoes were comparable to parent infected mosquitoes.

The dengue-1 candidate vaccine virus 45AZ5, was found to be phenotypically stable after up to 3 passages in Ae. aegypti or Ae. albopictus mosquitoes. Studies are continuing on its ability to infect, replicate in, and be transmitted by mosquitoes.

The dengue-4 candidate vaccine virus, H-241, Lot 1, was less efficient than its parent virus in infection and replication in both Ae. aegypti and Ae. albopictus mosquitoes. After oral infection, the vaccine virus replicated at a slower rate and to a lower titer than the parent virus in both mosquito species. Limited transmission data indicates the vaccine virus is either unable to or greatly reduced in its ability to be transmitted by mosquitoes compared to parent virus.

Increasing the extrinsic incubation temperature does not seem to increase the maximum attainable titer of the parent virus in mosquitoes but does decrease the time in which it is reached. In addition mosquitoes incubated at 33°C are more likely to develop disseminated infections of the secondary target organs than those incubated at 27°C.

The increase in temperature did not enhance vaccine virus replication. In contrast, rates of virus infection and dissemination in Ae. aegypti are reduced at higher temperatures, which may be the result of the ts nature of the vaccine virus.

Initial studies indicate that a primary infection with a dengue virus may interfere with replication of other strains of dengue virus. However, this primary infection does not effect replication of a related flavivirus, West Nile, or unrelated bunyavirus, La Crosse.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH)78-23, Revised 1978).

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I. Statement of the problem

The purpose of this research project is to determine if dengue parental and candidate vaccine viruses differ in their respective abilities to infect, to replicate in, and to be transmitted by Aedes aegypti and Aedes albopictus mosquitoes. Attenuated candidate vaccines and parental strains of dengue-1 and dengue-4 viruses were compared in their vector-virus interactions.

The second, and related, objective of this research project was to determine if attenuated vaccine strains revert to virulence after mosquito passage. Should a live dengue vaccine be capable of infecting and subsequently be transmitted by mosquitoes to a new vertebrate and should the vaccine revert to virulence as a consequence of mosquito passage, then a natural infection cycle could be initiated.

The rationale for this project was that the temperature sensitive (ts) vaccine strains of the dengue viruses which are attenuated for man will also be modified in one or more parameters of vector-virus interactions. The hypotheses are 1) the vaccine strains will be less capable than parental strains in vector infection, 2) vaccine strains will differ from parent strains in their mode of development, 3) the vaccine strains will be less efficiently transmitted than parent strains, and 4) that the small plaque ts mutant virus populations will remain stable upon passage in vector mosquitoes.

II. Background

Dengue is of great tactical significance to the military because large numbers of troops can become incapacitated in a short period of time. Attenuated dengue vaccines have been developed at WRAIR.

This project report will present data on two dengue-1 candidate vaccine virus strains and two dengue-4 strains.

The first dengue-1 candidate vaccine tested, TP-56, passage 28, was derived from a human serum isolate made during an epidemic on the island of Nauru in the South Pacific. It was passaged in fetal rhesus lung (FRhL) cells but not mutagenized. The TP-56 candidate vaccine is ts, small plaqued, and produces a low level viremia in rhesus monkeys. Its corresponding parent virus is dengue-1 parent P7. The second dengue-1 candidate vaccine virus tested was 45AZ5, Lot 1-82, Run 1. The corresponding parent strain tested is dengue-1, parent 2, No. 2, P8D6.

The two dengue-4 candidate vaccine viruses tested are H-241 and SGd6, 14d delayed. Dengue-4 vaccine virus strain, H-241, was derived from a human serum isolate passaged 1x in a monkey, 3x in Ae. albopictus mosquitoes, 35x in primary dog kidney (PDK) cells, cloned 3x in PDK cells and 3x in FRhL cells. The other dengue-4 candidate vaccine, SGd6, was a serum isolate from a viremic volunteer who received the dengue-4 H-241 vaccine, and then passaged 1x in LLC-MK_2 cells. The parent virus strain used to compare with the above candidate vaccines was derived from the same human isolate (H-241), passaged 6x in PDK cells, 2x in Ae. albopictus (C6/36) cells, and 1x in FRhL cells.

Ideally a vaccine should not produce viremia, but if it does, it is reasonable to expect that the vaccine strain will infect mosquitoes poorly and will be inefficiently transmitted. This was demonstrated with the 17D yellow fever vaccine (Roubaud et al., 1937; Whitman, 1939), French neurotropic yellow fever vaccine (Davis et al., 1932; Roubaud and Stefanopoulo, 1933; Peltier et al., 1939), mouse-adapted dengue type 1 (Sabin, 1948), African green monkey kidney-adapted dengue type 2 (Price, 1973), and attenuated Japanese encephalitis vaccine virus (Chen and Beaty, 1982). Sabin (1948) showed that attenuated dengue virus, passed through mosquitoes, did not revert to pathogenicity for man, and Chen and Beaty (1982) demonstrated that the attenuated Japanese encephalitis vaccine did not revert to mouse virulence after mosquito passage. Nor did mosquito passage result in phenotypic changes for the candidate dengue-2 vaccine (Miller et al., 1982).

Thus, even if the vaccine did develop sufficient viremia to infect vectors, there would be little likelihood that the virus would be transmitted or revert to virulence.

III. Approach

The working hypothesis was made that the ts candidate vaccine viruses and the parental wild-type viruses would behave differently in vector mosquitoes. To test this hypothesis the efficiency of oral infection of each parental and vaccine candidate strain was to be determined in dose response studies. Sequential 10-fold dilutions of the virus preparations were to be used to infect groups of a minimum of 10 sibling mosquitoes per dilution. Such studies would also provide information about the optimal infective dose for the transmission and pathogenesis studies; doses much greater than the threshold could obscure differences in infectivity between the vaccine and parental viruses.

Mosquitoes used to determine infection rates, extrinsic incubation periods, and rates of oral transmission were to be infected via engorgement on known titer blood-virus mixtures. Vector competence studies and especially dose-response studies are greatly facilitated by the use of artificial blood meals. In previous contract periods an efficient technique was developed to orally infect Aedes species mosquitoes with artificial meal systems (Miller et al., 1982).

Vector-virus interactions were to be further investigated using immunofluorescent (IF) techniques to localize antigen in situ in organ dissections and cryostat sections of infected mosquitoes. The sites of restriction of replication (if restriction exists) of the vaccine strains would be defined by the comparative IF studies of antigen development in organs of mosquitoes.

A further obstacle to assessment of vector competence has been the lack of a suitable laboratory animal to use to detect mosquito transmission of low passage or attenuated dengue viruses. Development of an in vitro assay which permitted assay of transmission by inoculation of collected mosquito saliva into recipient mosquitoes was a major advance (Beaty and Aitken, 1979). This technique facilitated transmission assays for viruses that did not cause observable morbidity or mortality in animals. Unfortunately, mosquitoes could not always be induced to engorge upon the artificial meal system used to capture the saliva. Refinement by Rossignol and Spielman (unpublished data) of a saliva capture technique using oil-charged capillaries (Hurlbut, 1966), provided a new in vitro technique to assay for virus transmission.

The combination of transmission and comparative pathogenesis studies and the determination of dose-response curves (thresholds of infection) were thought to be adequate to reveal differences in vector-virus interactions between parental and vaccine viruses.

In addition to the primary objectives stated above, the effect of different extrinsic incubation temperature on vector-virus interactions in orally infected mosquitoes was investigated. Studies were also initiated to determine if infection of a mosquito with one dengue virus type would interfere with infections of other dengue types, West Nile, a related flavivirus, or La Crosse, an unrelated bunyavirus.

IV. Materials and Methods

A. Viruses:

Dengue-1

Parent (P7) and vaccine (TP-56) dengue-1 stock viruses were prepared by inoculation of LLC-MK_2 cells. Viruses were harvested after 7 days, aliquoted, and frozen. Subsequently, stocks were prepared by inoculation of Aedes albopictus C6/36 cells. Viruses were harvested after 14 days (27°C), and used as fresh preparations. Parent (P8D6) and vaccine (45AZ5) dengue-1 stock viruses were prepared by inoculation of C6/36 cells initially rather than LLC-MK_2 cells.

Dengue-4

The original infected human serum isolate, H-241, was the source of the parental virus. Virus stocks of the parent and both derived

vaccine strains of dengue-4 virus were prepared by inoculation of C6/36 tissue culture cells with the respective seed virus. On day 14-17 post inoculation, cells and fluids were harvested, centrifuged, and the supernatant was aliquoted and frozen at -70°C. The viruses were passaged again in C6/36 cells for fresh virus preparations used in all experiments.

B. Mosquitoes:

Two vector species were used in these studies: the Puerto Rico strain of Ae. aegypti and the Oahu (Hawaii) strain of Ae. albopictus. Emerged adults were allowed to feed on sugar cubes and had access to water wicks. When this procedure was followed it was not necessary to starve mosquitoes prior to engorgement on infectious blood meals; generally greater than 80% of the mosquitoes exposed became fully engorged. The mosquitoes were maintained at either 27°C or 33°C and at approximately 75% relative humidity (RH).

C. Conjugate:

The anti-dengue-1 or -4 conjugate was prepared by hyper-immunization of mice (Brandt et al., 1967). Immunoglobulins were precipitated from the ascitic fluids with $(NH_4)_2SO_4$ and conjugated with fluorescein isothiocyanate (Spendlove, 1966; Hebert et al., 1972). Conjugated antibodies were purified by Sephadex G-50 column chromatography.

D. Virus Assay:

Titrations

All titrations were done using Lab-Tek slides seeded with Aedes albopictus C6/36 cells. Serial 10-fold dilutions of the preparations were inoculated into the plates. After 7 days incubation (28°C), slides were examined by immunofluorescence (IF) for the presence of viral antigen.

Antigen-detection

Immunofluorescence technique was used to localize viral antigen in situ in organ dissections and cryostat sections of mosquitoes (Beaty and Thompson, 1976, 1978) and in head and abdominal squash preparations (Kuberski and Rosen, 1977).

E. Oral Infection of Mosquitoes

Parental and vaccine viruses were each inoculated into flasks of Ae. albopictus C6/36 cells (27°C). After incubation periods of 14 - 17 days, cells were detached from the flasks with rubber policemen, and the cell fluid suspensions were centrifuged at 500xg for 10 minutes. The cell pellet was resuspended in a portion of the remaining fluid, and then equal parts of washed human or rabbit red blood cells and 10% sucrose in heat-inactivated calf serum were

added. Drops of this artificial blood meal were placed on the screening of cages holding mosquitoes. Engorged mosquitoes were removed and maintained at 27°C and 65-75% RH for 14-21 days.

F. Oral Transmission

After an extrinsic incubation period of 14-21 days mosquitoes were induced to salivate using oil-charged capillaries (approximately 0.05 ml).

Legs and wings were removed from mosquitoes and probisci were inserted into capillary pipettes charged with 3.5 ul of Cargille immersion oil. After 30 to 60 minutes exposure, mosquitoes were removed and the capillaries were placed in Eppendorf Centrifuge tubes containing 0.1 ml of 20% FCS-PBS diluent. Tubes were centrifuged to force virus into the diluent, which was subsequently inoculated into recipient mosquitoes. After incubation at 27°C for 14-17 days the recipient mosquitoes were head squashed and examined by IF. Detection of viral antigen in the head tissues of the recipient mosquitoes was interpreted as a transmission of dengue virus.

G. Dengue Interference Study

Ae. albopictus mosquitoes were divided into two groups, one group was intrathoracically inoculated with dengue-1 vaccine virus (TP-56) and the other used as uninfected controls. After 7 or 14 days incubation at 27°C, mosquitoes from both groups were challenged with a parent strain of dengue-1, dengue-2, dengue-4, West Nile, or La Crosse virus. The mosquitoes were incubated for an additional 14 days, harvested, and frozen at -70°C. Triturated samples were sent to Dr. Kenneth H. Eckels to be assayed.

V. Results

A. Dengue-1 studies

1. Dengue-1 candidate vaccine TP-56

Limited studies were conducted with the non-mutagenized dengue-1 candidate vaccine (TP-56). Communication with Dr. Kenneth H. Eckels indicated that this particular candidate vaccine strain was not attenuated in cell systems, and thus would not likely be an acceptable candidate vaccine. Studies in mosquitoes reported in the First Annual Report (January 1, 1983) also indicated that this was the case in mosquitoes.

a. Threshold of infection studies
See First Annual Report, January 1, 1983.

b. Growth curves
See First Annual Report, January 1, 1983

c. Oral transmission

To determine the ability to orally transmit virus, saliva samples were collected from Ae. albopictus mosquitoes infected with parent or vaccine virus. The parent blood meal titered 7.50 $\log_{10}$ $TCID_{50}$ per ml, and the vaccine meal titered 7.75 logs. After 21 days extrinsic incubation at 27°C, mosquitoes were processed by immunofluorescence for the presence of viral antigen. Infection and dissemination rates were 80% (16/20) for the parent virus and 100% (20/20) for the vaccine virus (Table 1). Saliva collected was stored at -70°C for 9 months before testing, which may have inactivated virus. However, 13% (2/16) parent infected mosquitoes transmitted virus, in contrast to only 5% (1/19) of those infected with vaccine virus.

Similar data was collected for Ae. albopictus mosquitoes extrinsically incubated at 27°C or 33°C. The parent and vaccine blood meals titered 7.50 and 7.25 $\log_{10}$ $TCID_{50}$ per ml respectively. Saliva samples were collected on days 7 and 14 post-feeding. Generally, infection and dissemination rates in both parent and vaccine temperature treatment groups increased with time (Table 2). After 7 days, infection and dissemination rates tended to increase faster when the mosquitoes were incubated at the higher temperature (33°C). Rates were approximately equal in both temperature treatment groups by Day 14 post-feeding. The vaccine virus again was found to be more efficient at infection and dissemination in the mosquito than the parent virus. Saliva samples collected from mosquitoes incubated 14 days and inoculated into recipient mosquitoes for assay, indicated that both parent and vaccine virus disseminated and were transmitted. Fifty percent (3/6) of the mosquitoes infected with parent virus and incubated at 27°C transmitted, while 67% (4/6) infected with vaccine virus transmitted. Parent and vaccine virus infected mosquitoes incubated at 33°C transmitted virus 50% (3/6) and 38% (3/8) of the time respectively (Table 2).

d. Pathogenesis

Selected mosquitoes engorging either parent or vaccine virus were dissected and major organ systems examined by immunofluorescence for evidence of infection (Table 3). At 7 days post-feeding the mosquitoes in both the 27°C and 33°C treatment groups had infections primarily limited to the midgut tissues. At 14 days, virus had disseminated from the midgut to other organs and the head tissues. Unfortunately a large number of organs in the 27°C treatment groups washed off the slide during the staining procedure. However with the limited amount of data available mosquitoes incubated at 33°C seemed to

contain more detectable antigen in a wider variety of tissues than vaccine or parent virus infected mosquitoes incubated at 27°C.

e. Reversion studies

Because this candidate vaccine virus was deemed inadequate as a vaccine, reversion studies were not conducted. However passaged mosquito suspensions are available for analysis.

2. Dengue-1 candidate vaccine 45AZ5

a. Threshold of infection studies

To determine the comparative ability of parent and vaccine viruses to infect both Ae. aegypti and Ae. albopictus mosquitoes. Each species was permitted to engorge blood meals containing serial 10-fold dilutions of the respective blood meals. After 21 days extrinsic incubation at 27°C, mosquitoes were stored at -70°C until processing. The mosquitoes are currently being examined by immunofluorescence for the presence of viral antigen.

b. Growth curves

To determine the comparative ability of parent and vaccine dengue-1 viruses to infect and to replicate in Ae. albopictus and Ae. aegypti mosquitoes, each species engorged infected blood meals. Mosquitoes were randomly separated after engorgement and held at 27°C or 33°C for the extrinsic incubation period. On selected days post-feeding mosquitoes engorging parent and vaccine virus were frozen at -70°C until processing.

Currently Ae. aegypti engorging parent or vaccine virus blood meals are being triturated and titrated in Lab-tek® slides for construction of a growth curve. These blood meals titered 7.50 and 7.25 $\log_{10}$ $TCID_{50}$ per ml respectively.

c. Reversion studies

To determine if the candidate vaccine reverted to parental phenotype, it was passaged three times by intrathoracic inoculation in both Ae. aegypti and Ae. albopictus mosquitoes. Suspensions were made and samples of the third passage were sent to Dr. Kenneth H. Eckels. The vaccine passage in each species was found to be small plaqued, and ts at the non-permissive temperature of 38.5°C.

B. Dengue-4 studies

1. Dengue-4 candidate vaccine H-241, Lot 1

The dengue-4 parent (human H-241 isolate) and the derived candidate vaccine were compared in their ability to infect and to replicate in Ae. albopictus and Ae. aegypti mosquitoes. During these studies, we were informed that the dengue-4 trials with human volunteers were disappointing. Only 2 of 5 inoculated volunteers sero-converted. An alternate virus, derived from an isolate taken from one of the human volunteers, was being considered as a vaccine.

a. Threshold of infection studies

To determine the comparative ability of the parent and vaccine viruses to infect Ae. albopictus and Ae. aegypti, mosquitoes were permitted to engorge sequential dilutions of the respective blood meals. After 21 days extrinsic incubation at 27°C, mosquitoes were processed by immunofluorescence for the presence of viral antigen.

Aedes albopictus mosquitoes

See First Annual Report, January 1, 1983.

Aedes aegypti mosquitoes

Infection and dissemination rates are shown in Table 4. The parent virus was markedly more efficient in oral infection of Ae. aegypti than the vaccine virus. The infection rates of mosquitoes ingesting ≥ 6.75 $\log_{10}$ $TCID_{50}$ per ml of parent virus was 86% (53/73). In contrast, only 10% (6/62) of the mosquitoes ingesting ≥ 7.0 logs of vaccine virus became infected. The median oral infectious dose (OID_{50}) for the parent virus was approximately 5.0 $\log_{10}$ $TCID_{50}$ per ml; the OID_{50} for the vaccine was >8.0 logs.

b. Growth curves

To determine the comparative ability of parent and vaccine dengue-1 viruses to infect and to replicate in Ae. albopictus and Ae. aegypti, mosquitoes were allowed to engorge infectious blood meals. If sufficient numbers engorged, mosquitoes were randomly separated into two groups and held at 27°C or 33°C for the extrinsic incubation period. On selected days post feeding, mosquitoes engorging parent or vaccine virus were frozen at -70°C until processing.

Aedes albopictus mosquitoes

In the previous contract period three separate studies were conducted with Ae. albopictus mosquitoes resulting in a growth curve of parent and vaccine virus in mosquitoes incubated at 27°C and a comparative growth curve of virus in mosquitoes incubated at 27°C and 30°C.

A fourth study was conducted with infected mosquitoes extrinsically incubated at 27°C or 33° C. This study would allow direct comparison between studies of Ae. albopictus and Ae. aegypti mosquitoes. The parent meal titered 9.0 $\log_{10}$ $TCID_{50}$ per ml. The vaccine meal titered 8.5 logs per ml. Titrations of mosquitoes have not been completed but partial data can be seen in Tables 5 and 6.

Aedes aegypti mosquitoes

For a comparison of the ability of the parent and vaccine dengue-4 viruses to infect and to replicate in Ae. aegypti, studies similar to those described above were conducted.

Trial 1

In the first trial, the parent meal titered 7.75 $\log_{10}$ $TCID_{50}$ per ml. The vaccine meal titered 8.0 logs per ml. The mosquitoes were extrinsically incubated at 27°C. The parent virus efficiently infected and replicated in mosquitoes (Table 7). By day 5 post-feeding 75% (3/4) mosquitoes tested were positive for parent virus. In contrast, vaccine virus was not detected until day 19 post-feeding. The overall infection rates for mosquitoes assayed between 5 and 21 days extrinsic incubation were 94% (30/32) for those engoraging the parent virus and 6% (2/32) for those engorging vaccine virus.

Trial 2

In the second trial, mosquitoes engorging parent and vaccine virus blood meals titering 7.50 and 6.75 $\log_{10}$ $TCID_{50}$ per ml respectively, were randomly separated into two groups. One group was extrinsically incubated at 27°C, the other at 33°C. Titers of individual mosquitoes incubated at 27°C are shown in Table 8, and those incubated at 33°C in Table 9.

The parent virus efficiently infected and replicated in mosquitoes. However an eclipse period as was seen in Trial 1 was not demonstrable. Seventy-five percent (3/4) of the mosquitoes contained significant amounts of parent virus on day 3 post-feeding. In contrast, mosquitoes

ingesting vaccine virus were found to contain virus only intermittently after day 3 post-feeding. The overall infection rates for mosquitoes processed between days 3 and 21 of extrinsic incubation were 83% (30/36) for those engorging the parent virus and 31% (11/36) for those engorging the vaccine virus.

The same phenomenon was noted when the mosquitoes were incubated at 33°C (Table 9). There was no discernable eclipse phase for mosquitoes ingesting parent dengue-4 virus; most contained significant titers of virus by 3 days extrinsic incubation. Again vaccine virus was only demonstrated in intermittent individuals throughout the extrinsic incubation period. The overall infection rates for mosquitoes incubated at 33°C and processed between 3 and 21 days extrinsic incubation were 86% (31/36) for those engorging parent virus and 33% (12/36) for those engorging vaccine virus.

Incubation of mosquitoes at 33°C tended to result in faster replication of the parent virus. Mosquitoes incubated at 33°C contained greater amounts of virus early in the infection as evidenced by their higher mean titers. However later in the extrinsic incubation period mean titers were similar for mosquitoes incubated at 27° and 33°C (Tables 8 and 9).

c. Oral transmission

To determine oral transmission capability, saliva samples were collected from Aedes albopictus and Aedes aegypti mosquitoes which had engorged dengue-4 parent or vaccine virus.

Aedes albopictus mosquitoes

Mosquitoes which engorged dengue-4 parent or vaccine blood meals containing 7.50 and 7.25 $\log_{10}$ $TCID_{50}$ per ml respectively, were extrinsically incubated at 27°C or 33°C. Saliva samples were collected on days 7 and 14 post-feeding.

None of the mosquitoes ingesting the vaccine virus blood meal contained detectable virus antigen after 7 or 14 days of incubation at either temperature (Table 10). Similarly none of the mosquitoes engorging parent virus and incubated at 27°C for 7 days contained detectable antigen. However, 30% (3/10) of the mosquitoes in the 33°C treatment were infected and 10% (1/10) had disseminated infections. After 14 days extrinsic incubation, infection and dissemination rates in both the 27°C and 33°C treatment groups were 70% and 100% respectively. Oral transmissions are to be determined for these treatment groups.

Aedes aegypti mosquitoes

Trial 1

Infection, dissemination, and transmission rates for Ae. aegypti mosquitoes orally infected with dengue-4 parent and vaccine viruses and extrinsically incubated at 27°C for 21 days are found in Table 11. The parent and vaccine blood meals titered 7.75 and 8.0 $\log_{10}$ $TCID_{50}$ per ml respectively. Infection and dissemination rates in mosquitoes engorging parent virus were 82% (9/11), in contrast to rates of 10% (2/20) for mosquitoes ingesting vaccine virus. Of the parent virus infected mosquitoes 56% (5/9) transmitted while those infected with vaccine failed to transmit.

Trial 2

Infection, dissemination, and transmission rates for Ae. aegypti mosquitoes orally infected with dengue-4 parent and vaccine viruses and extrinsically incubated at 27°C or 33°C for 14 or 21 days are found in Table 12. The parent and vaccine virus blood meals titered 7.50 and 6.75 $\log_{10}$ $TCID_{50}$ per ml respectively.

After 14 days mosquitoes engorging parent virus and incubated at 27°C or 33°C had infection rates of 85% (17/20) and 95% (19/20) respectively. Dissemination rates of the 33°C mosquitoes were higher (90% = 19/20) than those of the 27°C mosquitoes (70% = 14/20). Comparatively, mosquitoes engorging vaccine virus had lower infection and dissemination rates than parent infected mosquitoes incubated at the same temperatures. The greatest differences were seen in dissemination rates of 20% (4/20) at 27°C and 40% (8/20) at 33°C.

After 21 days, infection and dissemination rates in mosquitoes engorging parent virus were almost identical between and within treatment groups (Table 12). Mosquitoes engorging vaccine virus and incubated at 27°C had an infection rate comparable (90%) to parent infected mosquitoes but a lower dissemination rate. Those incubated at 33°C had considerably lower infection and dissemination rates, 60% (12/20) and 25% (5/20), when compared to the parental treatment group. Saliva samples are being inoculated into recipient mosquitoes to determine oral transmission rates.

d. Pathogenesis

Selected mosquitoes engorging either parent or vaccine virus were dissected and major organ systems examined by immunofluorescence for evidence of infection (Table 13). Unfortunately, many organs washed off the slides during the staining procedure. However, trends

were similar to results obtained before (See Results A-1-d). After 14 days most infections had disseminated from the midgut. Again parent infected mosquitoes incubated at 33°C seemed to contain a greater amount of viral antigen than those at 27°C.

e. Reversion studies

To determine if this candidate vaccine reverted to parental phenotype, it was passaged three times by intrathoracic inoculation in both Ae. albopictus and Ae. aegypti mosquitoes. Suspensions were made and samples of the third passage were sent to Dr. Kenneth H. Eckels. The vaccine passaged in Ae. albopictus mosquitoes was small plaqued and ts at the non-permissive temperature of 38.5°C. The vaccine passaged in Ae. aegypti apparently was of insufficient titer after shipment to W.R.A.I.R. from C.S.U. to be detected in Dr. Eckels system.

2. Dengue-4 candidate vaccine SGd6

Candidate vaccine virus SGd6 was derived from an isolate obtained from one of the human volunteers given the dengue-4 vaccine candidate H-241, Lot 1.

Vaccine stocks were prepared in C6/36 cell cultures as described in the Materials and Methods Section above. When titered, only a trace (<1.0 $\log_{10}$ $TCID_{50}$/ml) of vaccine virus was found. A second attempt to make new stocks met with similar results. A second passage of these new stocks also failed to produce any detectable virus.

Dr. Eckels was informed of the situation and we were told his laboratory had similar results in work with the vaccine. He suggested that the virus be passaged in mosquitoes to adapt it to C6/36 culture cells. After 3 passages in Ae. aegypti and Ae. albopictus mosquitoes we were still unable to detect virus. Dr. Eckels has informed us that we will be sent another dengue-4 candidate vaccine to test.

C. Dengue Interference Study

Aedes albopictus mosquitoes intrathoracically inoculated with dengue-1 vaccine virus (TP-56) were then challenged with dengue-1, 2, or 4 parent virus, or a related flavivirus, West Nile, or an unrelated bunyavirus, La Crosse. Mean virus titers and plaque morphology of infected mosquitoes are found in Table 14. Dengue-1 vaccine virus is small plaqued and ts. In the control mosquitoes all the wild type challenge viruses with the exception of dengue-2 were large or of mixed plaque size and grow at the non-permissive temperature (38.5°C) (Table 14). Mosquitoes challenged with a dengue virus 7 days after dengue-1 vaccine inoculation contained virus of small plaque morphology. If challenge was with West Nile

or La Crosse virus plaque sizes were mixed. Similar results were obtained in the group challenged at 14 days; however, the control mosquitoes inoculated with dengue-1 parent alone contained virus of only small plaque morphology. Data on the temperature sensitivity of these virus isolates are currently being collected by Dr. Eckels.

VI. Discussion

The dengue-1 candidate vaccine virus, TP-56, was not altered in its ability to infect and to replicate in Aedes aegypti or Aedes albopictus mosquitoes. Oral infection and dissemination rates with the vaccine virus were as high or higher than for the parent virus (Tables 1 and 2). Pathogenesis data (Table 3) is limited, but Ae. albopictus mosquitoes incubated at 33°C tend to contain more detectable antigen. Early in the infection (7 days) antigen is primarily limited to the midgut tissues, later (14 days) the virus disseminates to a wider variety of tissues. Since this vaccine is no longer considered a candidate, no further work will be done with it. Nonetheless, on the basis of its ability to orally infect, replicate in, disseminate, and be transmitted by mosquitoes, this candidate vaccine would not seem to be sufficiently attenuated to preclude mosquito infection during engorgement on viremic vaccines.

The dengue-1 candidate vaccine virus, 45AZ5, was found to be phenotypically stable after up to 3 passages in Ae. aegypti or Ae. albopictus mosquitoes. Studies are continuing on its ability to infect, replicate in, and be transmitted by mosquitoes.

The dengue-4 candidate vaccine virus, H-241, Lot 1, was less efficient than its parent virus in infection and replication in both Ae. aegypti and Ae. albopictus mosquitoes. The results are similar to those reported in studies comparing dengue-2 parent and candidate vaccine viruses (Miller et al., 1983). In the previous contract period, the OID_{50} of the vaccine virus in Ae. albopictus mosquitoes was >7.0 $\log_{10}$ $TCID_{50}$ per ml and 4.6 logs for the parent virus. In Ae. aegypti mosquitoes (Table 4), the OID_{50} for those mosquitoes engorging vaccine virus was >8.0 $\log_{10}$ $TCID_{50}$ per ml. and 5.0 logs for parent virus. Comparison of the OID_{50}'s for the parent infected mosquitoes would indicate that Ae. albopictus is the more competent mosquito species. However, it must be noted that these observations were made using highly adapted laboratory mosquito strains. Caution must be observed when extrapolating data derived from these two laboratory strains to species differences in vector competence in nature.

In both mosquito species, after oral infection, the vaccine virus replicated at a slower rate and to a lower titer than the parent virus. The majority of the data on Ae. albopictus mosquitoes were included in the First Annual Report. However, incomplete data collected for growth curves in this mosquito species incubated at 27°C and 33°C indicates a dose response of the mosquitoes to the dengue-4 viruses (Table 5 and 6). Previously, mosquitoes

engorging parent blood meals titering 7.0 logs had eclipse periods early in infections in which no virus could be demonstrated (First Annual Report, Table 5). However, with a significant increase in blood meal titer (9.0 $\log_{10}$ $TCID_{50}$ per ml) the eclipse period is no longer observed (Table 5). The effect of increased blood meal titer on infection rates in mosquitoes ingesting vaccine virus is less evident, but there does seem to be some correlation between titer and infection rate

As observed with Ae. albopictus mosquitoes, Ae. aegypti mosquitoes infected with the parent virus and incubated at 27°C, exhibited an eclipse period early in the infection which was decreased or eliminated with an increase in extrinsic incubation to 33°C (Tables 7, 8, and 9). The increase in temperature does not seem to increase the maximum titer of the virus in mosquitoes but does decrease the time in which it is reached. In addition, mosquitoes incubated at 33°C are more likely to develop disseminated infections of the secondary target organs than those incubated at 27°C (Tables 2, 11, 12, and 13). These studies confirm previously reported results with this and other dengue viruses tested (First Annual Report) and by Watts and co-workers (1980) describing increased transmission rates in Ae. aegypti incubated at 30°C.

In contrast, increased extrinsic incubation temperature did not enhance vaccine virus replication. Rates of virus infection and dissemination in Ae. aegypti were reduced at higher temperatures (Table 12). This may be the result of the ts nature of the vaccine virus, although the temperature used in these studies are far below the non-permissive temperature of the vaccines (38.5°C).

During the course of these studies, we were informed by Dr. Kenneth H. Eckels that clinical trials of this candidate vaccine were disappointing and that it is no longer considered a candidate. Thus, oral transmission trials have not been completed. Transmission data is limited but Table 11 does indicate that the vaccine virus is unable or is markedly less efficient in its ability to be transmitted by Ae. aegypti mosquitoes, than the parent virus. If time permits oral transmission trials will be concluded.

However, on the basis of the marked attenuation of the vaccine virus for vector infection and replication and its stable phenotype this candidate vaccine would seem to be sufficiently attenuated to preclude mosquito infection by engorgement on recent vaccinees. Furthermore, even in the event that mosquito infection did occur, subsequent transmission of the virus would seem unlikely due to the reduced ability of the vaccine to replicate in mosquitoes.

The new dengue-4 candidate vaccine (SGd6), did not replicate in our C6/36 cell cultures on which our testing system is based. Multiple attempts have been made to passage the virus in C6/36 cells and in adult mosquitoes, but they have been unsuccessful.

Similar results have been observed by Dr. Eckels in his laboratory. We have been informed that another dengue candidate vaccine will be forthcoming to replace this one.

Apparently previous infection with dengue-1 vaccine virus interferes with the subsequent replication of dengue-1 and dengue-4 virus strains. However, it does not interfere with the replication of West Nile virus, a related flavivirus, or La Crosse virus an unrelated bunyavirus. Since dengue-2 challenge virus is also small plaqued it is unknown whether interference occurred. Studies are currently in progress to assay these mosquitoes at nonpermissive temperature for the candidate dengue-1 vaccine. If no plaques are detected at the nonpermissive temperature, then infection with dengue-1 virus would also seem to interfere with superinfection by dengue-2 virus.

VII. Conclusions

A. The dengue-1 candidate vaccine virus (TP-56, non-mutagenized):

1. Is not attenuated in its ability to infect and to replicate in mosquitoes but rather is more efficient in both parameters than its parent virus.

2. Is not sufficiently attenuated to preclude vector infection during engorgement on viremic vaccines.

3. Is not sufficiently attenuated in its ability to replicate in vectors to minimize the risk of transmission.

B. The dengue-4 candidate vaccine virus (H-241, Lot 1):

1. Is markedly less efficient than its parent in oral infection of and replication in Aedes albopictus and Aedes aegypti mosquitoes.

2. Is sufficiently attenuated for oral infection of mosquitoes to reduce the possibility of vector infection during engorgement on recent vaccines.

3. Is sufficiently attenuated in its ability to replicate in mosquitoes that its potential for transmission by infected mosquitoes would be reduced.

4. Is phenotypically stable after multiple mosquito passages.

C. Dengue Interference

Limited data suggests that a previous infection with a particular dengue virus precludes the replication of other dengue viruses. However, other related flaviviruses (West Nile) and unrelated bunyaviruses (La Crosse) are not effected.

VIII. Publications

The following article detailing the results of the comparisons of the dengue-2 candidate vaccine and its parent virus in Aedes aegypti was published in 1982.

Miller, B. R., Beaty, B. J., Aitken, T. H. G., Eckels, K. H., and Russell, P. K. 1982. Dengue-2 vaccine: oral infection, transmission, and lack of evidence for reversion in the mosquito, Aedes aegypti. Am. J. Trop. Med. Hyg., 31:1232-1237.

Three manuscripts are currently in preparation and are to be submitted for publication in 1984. Tentative titles are:

1. Dengue-4 candidate vaccine virus: Comparisons of growth, replication and transmission in Aedes aegypti and Aedes albopictus mosquitoes.

2. Fluorescent foci titrations of dengue virus in microtiter plates: A fast, reliable, and inexpensive alternative.

3. Effects of different extrinsic incubation temperatures on virus infection, replication and transmission in mosquitoes.

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Table 1. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue-1 parent or vaccine virus (TP-56)[1] and extrinsically incubated for 21 days at 27°C.

	Parent	Vaccine
INFECTION	16/20 (80)[2]	20/20 (100)
DISSEMINATION	16/20 (80)	20/20 (100)
TRANSMISSION[3]	2/16 (13)	1/19 (5)

[1] Blood meal titers: Parent virus 7.50 Log_{10} $TCID_{50}$/ml
Vaccine virus 7.75 Log_{10} $TCID_{50}$/ml

[2] Number positive/total (%)

[3] Samples stored 9 months before processing which may have inactivated virus.

Table 2. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue-1 parent or vaccine virus (TP-56)[1] and extrinsically incubated at 27°C or 33°C.

		Parent		Vaccine	
		7 Days[2]	14 Days	7 Days	14 Days
27°C	INFECTION	3/15 (20)[3]	11/19 (58)	5/15 (33)	17/20 (85)
	DISSEMINATION	0/15 (0)	6/19 (32)	2/15 (13)	10/20 (50)
	TRANSMISSION[1]	-	3/6 (50)	TBA[5]	4/6 (67)
		7 Days	14 Days	7 Days	14 Days
33°C	INFECTION	10/15 (67)	14/20 (70)	12/15 (80)	14/20 (70)
	DISSEMINATION	2/15 (13)	13/20 (65)	3/15 (20)	12/20 (60)
	TRANSMISSION	TBA	3/6 (50)	TBA	3/8 (38)

[1] Blood meal titers: Parent virus 7.50 Log_{10} $TCID_{50}$/ml
Vaccine virus 7.25 Log_{10} $TCID_{50}$/ml

[2] Days extrinsic incubation post-feeding

[3] Number positive/number tested (%)

[4] Number postive/number tested (%)

[4] Only samples containing discernable mosquito saliva were assayed.

[5] To be assayed.

Table 3. Pathogenesis of dengue-1 parent and vaccine (TP-56) viruses in orally infected Aedes albopictus mosquitoes extrinsically incubated for 7 or 14 days at 27°C or 33°C.

		7 Days Organs[1]								14 Days Organs							
Virus[2]	Temp	Mosq	HD	GN	SG	OVD	HT	FG	MG	Temp	Mosq	HD	GN	SG	OVD	HT	FG	MG
Parent	27°C	4	-	-	-	-	-	-	2+	27°C	1	1+	M	M	M	M	M	M
		9	-	M[2]	-	-	-	-	1+		2	M	M	M	M	M	M	M
		10	-	-	M	-	-	-	-		3	M	M	M	M	M	M	M
		12	-	-	-	-	M	+	3+		4	-	-	M	-	M	-	-
		14	-	M	-	-	M	-	-		5	-	M	M	M	M	2+	2+
	33°C	2	-	-	-	-	-	-	-	33°C	1	4+	M	2+	1+	2+	3+	3+
		3	-	-	-	-	M	-	2+		2	4+	3+	2+	1+	M	3+	4+
		9	-	M	-	1+	M	-	4+		3	3+	1+	3+	2+	M	2+	3+
		10	1+	M	+	-	M	M	2+		4	4+	M	2+	2+	2+	M	3+
		14	-	-	-	M	-	-	1+		5	2+	3+	3+	M	1+	2+	3+
Vaccine	27°C	3	-	-	-	-	-	-	-	27°C	1	-	M	M	M	M	2+	3+
		4	3+	-	-	-	1+	-	2+		2	3+	M	2+	M	M	2+	2+
		7	-	-	-	-	-	-	-		3	-	M	-	-	-	M	3+
		10	-	-	-	-	-	-	2+		4	-	M	M	M	M	M	2+
		13	-	-	-	-	M	-	2+		5	2+	M	M	2+	M	2+	3+
	33°C	2	-	M	M	M	M	-	2+	33°C	1	3+	4+	3+	2+	M	M	M
		4	-	M	-	-	-	-	3+		2	3+	M	2+	1+	4+	3+	2+
		12	-	-	-	-	-	-	1+		3	3+	4+	2+	3+	M	3+	4+
		13	1+	-	-	1+	-	2+	3+		4	2+	M	M	-	M	2+	3+
		14	-	-	-	-	-	-	2+		5	3+	M	3+	M	M	2+	3+

[1] HD = head, GN = ganglion, SG = salivary glands, OVD = ovaries and ducts, HT = heart, FG = foregut, and MG = midgut

[3] Blood meal titers: Parent virus 7.50 $\log_{10}$ $TCID_{50}$/ml

Vaccine virus 7.25 $\log_{10}$ $TCID_{50}$/ml

[2] M = missing

Table 4. Infection rates of Aedes aegypti mosquitoes orally infected with graded doses of dengue-4 parent and vaccine (H-241, Lot 1) viruses after 21 days extrinsic incubation at 27°C.

Dilution of blood meal[1]	Parent virus		Vaccine virus	
	Infected(%)[2]	Disseminated(%)[3]	Infected(%)	Disseminated(%)
10^{0}	9/11 (82)	9/11 (82)	4/25 (16)	4/25 (16)
10^{-1}	54/62 (87)	54/62 (87)	2/37 (5)	2/37 (5)
10^{-2}	20/42 (48)	20/42 (48)	0/13 (0)	0/13 (0)
10^{-3}	4/24 (17)	3/24 (13)	0/39 (0)	0/39 (0)
Totals	87/139 (63)	86/139 (62)	6/114 (5)	6/114 (5)

[1] Parent virus titer 7.75 $\log_{10}$ $TCID_{50}$/ml
Vaccine virus titer 8.0 $\log_{10}$ $TCID_{50}$/ml

[2] Number mosquitoes positive for dengue-4 viral antigen in midgut/number tested

[3] Number mosquitoes positive for dengue-4 viral antigen in head tissues/number tested

Table 5. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 27°C.

Virus[1]	Mosquito	Days Post-Feeding 0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.5	3.5	≥5.5	4.5	4.5	4.25	5.0	3.75	≥5.25	4.75
	2	≥5.5	2.0	4.75	4.75	4.5	4.50	≥5.25	4.5	4.75	5.0
	3	5.0	3.5	≥5.5	≥5.5	4.5	4.50	3.5	3.0	3.75	≥5.5
	4	5.0	0	3.25	5.0	≥5.25	2.0	3.5	3.5	3.25	4.0
Vaccine	1	5.0	0	0	Tr	0	0	3.25	2.75	0	0
	2	4.25	0	Tr[2]	Tr	3.0	0	2.75	0	0	Tr
	3	5.0	0	0	2.75	2.25	0	0	≥5.25	0	0
	4	4.5	0	0	2.75	3.25	2.25	0	0	3.25	0

[1] Blood meal titers: Parent virus 9.0 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.5 $\log_{10}$ $TCID_{50}$ per ml

[2] Tr = trace

Table 6. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes albopictus mosquitoes after oral infection and extrinsic incubation at 33°C.

Virus[1]	Mosquito	Days Post-Feeding 0	3	5	7	9	11	14	17	19	21
Parent	1	≥5.5	TBA	TBA	TBA	TBA	TBA	TBA	4.0	≥5.25	≥5.25
	2	≥5.5	TBA	TBA	TBA	TBA	TBA	TBA	≥5.5	0	≥5.5
	3	5.0	TBA	TBA	TBA	TBA	TBA	TBA	≥5.5	>5.5	≥5.5
	4	5.0	TBA	TBA	TBA	TBA	TBA	TBA	≥4.5	≥5.5	5.5
Vaccine	1	5.0	TBA	TBA	TBA	TBA	TBA	TBA	0	1.75	0
	2	4.25	TBA	TBA	TBA	TBA	TBA	TBA	0	0	0
	3	5.0	TBA	TBA	TBA	TBA	TBA	TBA	5.0	0	4.5
	4	4.5	TBA	TBA	TBA	TBA	TBA	TBA	0	3.5	0

[1] Blood meal titers: Parent virus 9.0 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.5 $\log_{10}$ $TCID_{50}$ per ml

[2] TBA = to be assayed

Table 7. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 27°C.

Virus[1]	Mosquito	Days Post-Feeding 0	3	5	7	9	11	14	17	19	21
Parent	1	3.5	0	3.25	2.0	2.0	3.25	≥3.5	≥3.0	4.0	3.5
	2	4.5	0	Tr[2]	2.5	2.0	≥3.25	0	2.75	4.0	4.75
	3	3.75	0	2.5	Tr	2.25	≥3.5	2.25	≥3.5	3.5	3.25
	4	3.5	0	0	Tr	3.0	3.0	2.75	≥3.5	4.0	3.75
Vaccine	1	4.75	0	0	0	0	0	0	0	3.0	0
	2	4.25	0	0	0	0	0	0	0	0	0
	3	3.5	0	0	0	0	0	0	0	2.0	0
	4	3.75	0	0	0	0	0	0	0	0	0

[1] Blood meal titers: Parent virus 7.75 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 8.0 $\log_{10}$ $TCID_{50}$ per ml

[2] Tr = trace

Table 8. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 27°C.

Virus[1]	Mosquito	Days Post-Feeding 0	3	5	7	9	11	14	17	19	21
Parent	1	3.5	4.0	4.25	2.75	2.0	4.75	4.25	0	4.25	3.5
	2	3.5	2.0	2.25	2.75	0	3.75	3.75	4.5	3.75	5.0
	3	3.25	Tr[2]	0	3.5	0	3.5	≥5.5	3.75	4.25	2.75
	4	3.25	0	0	2.5	3.75	3.5	5.0	≥4.5	2.5	4.75
Vaccine	1	4.0	0	2.0	0	0	2.5	0	0	≥5.5	4.0
	2	4.25	0	0	0	0	0	0	0	3.0	≥5.25
	3	3.5	0	0	0	0	0	2.0	0	0	0
	4	3.5	0	0	0	2.5	0	0	3.5	Tr	2.5

[1] Blood meal titers: Parent virus 7.5 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 6.75 $\log_{10}$ $TCID_{50}$ per ml

[2] Tr = trace

Table 9. Replication of dengue-4 parent and vaccine (H-241, Lot 1) viruses in Aedes aegypti mosquitoes after oral infection and extrinsic incubation at 33°C.

Virus[1]	Mosquito	Days Post-Feeding 0	3	5	7	9	11	14	17	19	21
Parent	1	3.5	3.0	≥5.0	2.5	4.5	≥5.25	≥5.0	4.0	4.75	4.5
	2	3.5	0	3.5	≥5.0	4.0	≥5.25	4.25	4.0	0	≥5.0
	3	3.25	3.25	4.25	3.5	4.5	5.0	0	3.75	3.75	≥5.5
	4	3.25	2.75	3.5	0	3.75	5.0	0	4.0	4.5	4.5
Vaccine	1	4.0	0	0	Tr[2]	0	0	≥5.25	0	0	0
	2	4.25	0	0	4.0	0	0	0	4.5	4.0	4.5
	3	3.5	0	2.5	0	0	0	≥5.5	Tr	0	0
	4	3.5	4.25	0	0	0	0	≥4.5	0	0	Tr

[1] Blood meal titers: Parent virus 7.5 $\log_{10}$ $TCID_{50}$ per ml
Vaccine virus 6.75 $\log_{10}$ $TCID_{50}$ per ml

[2] Tr = trace

Table 10. Infection, dissemination, and transmission rates of Aedes albopictus mosquitoes engorging a blood meal of dengue 4 parent or vaccine (H-241, Lot 1) virus[1] and extrinsically incubated for 7 or 14 days at 27°C or 33°C.

		Parent		Vaccine	
		7 Days[2]	14 Days	7 Days	14 Days
27°C	INFECTION	0/10 (0)[3]	7/10 (70)	0/10 (0)	0/10 (0)
	DISSEMINATION	0/10 (0)	7/10 (70)	0/10 (0)	0/10 (0)
	TRANSMISSION[1]	-	TBA[4]	-	-
		7 Days	14 Days	7 Days	14 Days
33°C	INFECTION	3/10 (30)	10/10 (100)	0/10 (0)	0/7 (0)
	DISSEMINATION	1/10 (10)	10/10 (100)	0/10 (0)	0/7 (0)
	TRANSMISSION	TBA	TBA	-	-

[1] Blood meal titers: Parent virus 7.50 Log_{10} $TCID_{50}$/ml
Vaccine virus 7.25 Log_{10} $TCID_{50}$/ml

[2] Days extrinsic incubation post-feeding

[3] Number positive/number tested (%)

[4] To be assayed.

Table 11. Infection, dissemination, and transmission rates of Aedes aegypti mosquitoes orally infected with dengue-4 parent and vaccine (H-241, Lot 1) viruses[1] and extrinsically incubated at 27°C for 21 days.

	Parent Virus	Vaccine virus
INFECTED	9/11 (82)[2]	2/20 (10)
DISSEMINATED	9/11 (82)	2/20 (10)
TRANSMITTING	5/9 (56)	0/2 (0)

[1] Blood meal titers: Parent virus 7.75 $\log_{10}$ $TCID_{50}$/ml

Vaccine virus 8.0 $\log_{10}$ $TCID_{50}$/ml

[2] Number positive/number tested (%)

Table 12. Infection, dissemination, and transmission rates of Aedes aegypti mosquitoes engorging a blood meal of dengue-4 parent or vaccine (H-241, Lot 1) virus[1] and extrinsically incubated at 27°C or 33°C for 14 or 21 days.

		Parent		Vaccine	
		14 Days[2]	21 Days	14 Days	21 Days
27°C	INFECTION	17/20 (85)[3]	19/20 (95)	13/20 (65)	18/20 (90)
	DISSEMINATION	14/20 (70)	17/20 (85)	4/20 (20)	10/20 (50)
	TRANSMISSION	TBA[4]	TBA	TBA	TBA
33°C	INFECTION	19/20 (95)	19/20 (95)	12/20 (60)	12/20 (60)
	DISSEMINATION	19/20 (95)	19/20 (95)	8/20 (40)	5/20 (25)
	TRANSMISSION	TBA	TBA	TBA	TBA

[1] Blood meal titers: Parent virus 7.50 Log_{10} $TCID_{50}$/ml
Vaccine virus 6.75 Log_{10} $TCID_{50}$/ml

[2] Days extrinsic incubation post-feeding

[3] Number positive/number tested (%)

4 To be assayed.

Table 13. Pathogenesis of dengue-4 parent and (H-241, Lot 1) vaccine viruses in Aedes aegypti mosquitoes after oral infection and incubation for 14 or 21 days at 27° and 33°C.

		14 Days Organs[1]							21 Days Organs						
Virus[2]	Temp.	Mosq	HD	GN	SG	OVD	MG	HG	Temp.	Mosq	HD	GN	SG	OVD	MG	HG
Parent	27°C	1	-	-	M[3]	-	3+	M	27°C	1	2+	M	M	-	3+	M
		3	2+	-	M	M	4+	-		2	1+	M	M	-	3+	M
		7	-	-	M	-	M	-		4	1+	M	-	-	3+	M
		10	2+	M	3+	-	M	M		16	2+	M	M	-	M	M
		15	2+	M	M	-	2+	M		19	2+	M	M	1+	4+	M
	33°C	3	4+	3+	4+	2+	M	M	33°C	1	3+	M	M	-	4+	-
		5	3+	3+	3+	2+	4+	1+		3	3+	M	3+	-	M	M
		7	3+	3+	5+	M	3+	-		4	4+	M	M	M	4+	1+
		8	-	M	M	M	M	-		8	4+	4+	4+	M	M	M
		14	4+	3+	3+	1+	M	M		13	4+	M	M	M	M	M
Vaccine	27°C	1	-	-	M	-	M	M	27°C	2	-	M	M	-	2+	-
		3	2+	-	M	-	3+	M		3	-	-	M	-	-	M
		9	3+	-	M	1+	3+	M		6	1+	-	M	M	3+	M
		13	-	M	-	-	2+	-		9	-	M	M	-	-	M
		16	-	M	M	1+	2+	M		10	1+	1+	1+	-	-	M
	33°C	2	3+	1+	3+	1+	3+	M	33°C	16	-	M	M	M	M	M
		4	-	-	M	-	4+	-		17	-	M	M	-	2+	-
		5	-	-	M	-	M	M		18	-	-	M	-	-	M
		7	2+	2+	4+	-	1+	-		19	-	M	M	-	-	M
		18	2+	M	M	-	M	M		20	-	-	M	-	M	-

[1] HD = head, GN = ganglion, SG = salivary glands, OVD - ovaries and ducts, MG = midgut, and HG = hindgut

[2] Blood meal titers: Parent virus 9.0 $\log_{10}$ $TCID_{50}$/ml

Vaccine virus 8.5 $\log_{10}$ $TCID_{50}$/ml.

[3] M - missing

Table 14. Dual infections of Aedes albopictus mosquitoes intrathoracically inoculated with dengue-1 vaccine (TP-56) and challenged 7 or 14 days later.

			Challenge Virus				
		Control	Dengue-1 parent	Dengue-2 parent	Dengue-4 parent	West Nile	La Crosse
DAY 7	Control	0	4.0×10^3 (Mix)[2]	2.1×10^3 (Small)	2.2×10^3 (Mix)	5.1×10^4 (Mix)	6.1×10^2 (Mix)
	Dengue-1 vaccine	4.5×10^3 (Small)	8.6×10^3 (Small)	9.3×10^3 (Small)	1.8×10^4 (Small)	$\geq 6.6 \times 10^4$ (Mix)	$4.4 \times \pm 0^3$ (Mix)
DAY 14	Control	0	3.8×10^3 (Small)	1.1×10^3 (Small)	2.0×10^2 (Large)	$\geq 8.2 \times 10^4$ (Mix)	7.0×10^2 (Mix)
	Dengue-1 vaccine	$\geq 3.1 \times 10^4$ (Small)	1.0×10^4 (Small)	2.1×10^3 (Small)	1.3×10^4 (Small)	1.3×10^4 (Mix)	6.1×10^3 (Mix)

[1] Mean titer (Pfu/ml) of five mosquitoes assayed.

[2] Plaque morphology of virus assayed.

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